

Downregulation of Diacylglycerol Kinase Delta Contributes to Hyperglycemia-Induced Insulin Resistance

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SUMMARY

Type 2 (non-insulin-dependent) diabetes mellitus is a progressive metabolic disorder arising from genetic and environmental factors that impair beta cell function and insulin action in peripheral tissues. We identified reduced diacylglycerol kinase δ (DGK δ) expression and DGK activity in skeletal muscle from type 2 diabetic patients. In diabetic animals, reduced DGK δ protein and DGK kinase activity were restored upon correction of glycemia. DGK δ haploinsufficiency increased diacylglycerol content, reduced peripheral insulin sensitivity, insulin signaling, and glucose transport, and led to age-dependent obesity. Metabolic flexibility, evident by the transition between lipid and carbohydrate utilization during fasted and fed conditions, was impaired in DGK δ haploinsufficient mice. We reveal a previously unrecognized role for DGK δ in contributing to hyperglycemia-induced peripheral insulin resistance and thereby exacerbating the severity of type 2 diabetes. DGK δ deficiency causes peripheral insulin resistance and metabolic inflexibility. These defects in glucose and energy homeostasis contribute to mild obesity later in life.

INTRODUCTION

Type 2 diabetes constitutes a burgeoning epidemic, which is expected to afflict over 300 million individuals world-wide by 2015 (Zimmet et al., 2001). Characteristic features include glucose intolerance, insulin resistance, hyperglycemia, and often hyperinsulinemia (Biddinger and Kahn, 2006). Skeletal muscle insulin

resistance and impaired beta cell function (Eriksson et al., 1989), arising from a combination of genetic and environmental factors (Florez et al., 2003; Laakso, 2005), are necessary for the development of type 2 diabetes. Although available therapies fail to completely normalize glycemia and insulin sensitivity in the long-run (Moller, 2001), aggressive control of hyperglycemia partly improves whole-body insulin sensitivity and skeletal muscle glucose uptake (Rossetti et al., 1990; Zierath et al., 1994) and attenuates the development of chronic complications such as retinopathy, nephropathy, and neuropathy (UK Prospective Diabetes Study Group, 1998).

Diacylglycerol (DAG) plays a role in lipid metabolism as a precursor of triglycerides and phospholipids and acts as a second messenger in cellular signaling. In rodents, glucose-induced insulin resistance is associated with a temporal increase in intracellular DAG mass (Kraegen et al., 2006). A persistent increase in intracellular DAG promotes intracellular lipid accumulation and aberrant signal transduction through activation of protein kinase C (PKC) isoforms and insulin resistance (Itani et al., 2002; Montell et al., 2001). The underlying mechanism for DAG-induced insulin resistance is incompletely understood, but increased PKC-mediated serine phosphorylation of the insulin receptor (IR) and insulin receptor substrate 1 (IRS-1) and subsequent downregulation of glucose transport are implicated (Yu et al., 2002). DAG is also phosphorylated to form phosphatidic acid (PA) to act as a lipid second messenger to regulate signals involved in metabolic and mitogenic responses, including phosphatidylinositol 4-phosphate 5-kinase, PKC ζ , and the mammalian target of rapamycin (Avila-Flores et al., 2005; Luo et al., 2004). Intracellular DAG and PA levels are tightly controlled, and disturbances in this balance may impair cellular metabolism.

Phosphorylation of DAG to PA is catalyzed by diacylglycerol kinases (DGK), a family of enzymes acting as key attenuators of DAG signaling (Kano et al., 2002). DGK isoforms are of

relevance for insulin action because DAG accumulation is associated with insulin resistance and impaired glucose uptake in skeletal muscle (Montell et al., 2001). Ten mammalian DGK isoforms have been classified into five subgroups based on primary structure (Sakane et al., 2007). DGK isoforms including α , δ , ε , and ζ are expressed in skeletal muscle, suggesting isoform-specific function (Topham, 2006; van Blitterswijk and Houssa, 2000). DGKs play a critical role in depleting DAG/increasing PA at specific intracellular sites, including plasma membrane, endoplasmic reticulum, Golgi apparatus, and nuclei (Nagaya et al., 2002; Topham et al., 1998). The direct role of DGK isoforms in the development of insulin sensitivity and diabetes mellitus is unknown.

We used oligonucleotide microarrays to identify defects in skeletal muscle from type 2 diabetic patients by examining gene-expression profiles (E-MEXP-1270). This revealed that DGK δ is downregulated in skeletal muscle from poorly controlled type 2 diabetic patients (Table S1 available online). DGK δ was originally cloned from a human testis library and is highly expressed in skeletal muscle (Sakane et al., 1996). A previous microarray analysis of *vastus lateralis* muscle from individuals participating in the HERITAGE Family Study shows that DGK δ expression was increased in people who exhibited enhanced insulin sensitivity after exercise training (Teran-Garcia et al., 2005), suggesting that increased skeletal muscle DGK δ expression may be a marker of improved insulin action.

We show that DGK δ protein expression and total DGK activity are reduced in skeletal muscle from type 2 diabetic patients and diabetic rodents and normalized upon correction of hyperglycemia. Reduced DGK δ activity, either by chemical inhibition or by genetic ablation of the enzyme using DGK δ haploinsufficient mice, causes aberrant insulin signaling, glucose uptake defects in skeletal muscle and adipose tissue, impaired whole-body insulin sensitivity, and obesity. Our findings suggest a previously unrecognized role for DGK δ hyperglycemia-induced peripheral insulin resistance. Reduced DGK δ may exacerbate the severity of type 2 diabetes.

RESULTS

Skeletal Muscle DGK δ Protein Content and Activity Is Reduced in Type 2 Diabetes

We determined DGK δ protein expression in lysates prepared from *vastus lateralis* skeletal muscle biopsies obtained from 11 type 2 diabetic and 11 nondiabetic participants with fasting blood glucose of 9.9 ± 1.2 and 5.3 ± 0.2 mmol/l, respectively. The clinical characteristics for this cohort are presented in Table S2. Skeletal muscle DGK δ protein expression was reduced 37% (Figure 1A; $p < 0.05$) in type 2 diabetic subjects, while DGK α , ε , and ζ protein was unaltered (Figure 1B). Total skeletal muscle DGK activity was markedly decreased in type 2 diabetic patients (Figure 1C), suggesting that DGK δ is the predominantly expressed DGK isoform contributing to total DGK activity in human skeletal muscle. Skeletal muscle DGK δ protein expression was negatively correlated with fasting blood glucose ($r = -0.50$; $p < 0.01$) and HbA $_{1c}$ ($r = -0.60$; $p < 0.05$) and tended to correlate with body mass index ($r = -0.31$; $p = 0.08$) and whole-body glucose utilization ($r = 0.43$; $p = 0.06$), but not fasting

insulin or percentage body fat (Figure S1). In a similar cohort of type 2 diabetic patients, skeletal muscle IR, IRS-1, IRS-2, MAP kinase, glycogen synthase, and glucose transporter 4 (GLUT4) protein content was unchanged, despite impairments in insulin signaling and glucose uptake (Krook et al., 2000).

We hypothesized that DGK δ protein expression may be directly related to the level of glycemia. We determined whether hyperglycemia directly downregulates DGK δ protein expression in cultured human skeletal muscle from 5 nondiabetic participants incubated with either 5.5 mM or 25 mM glucose for 96 hr (Figure 1D). DGK δ protein expression was downregulated in myotubes exposed to 25 mM glucose, while protein expression of DGK ζ was unaltered. To assess whether changes in glycemia regulate DGK δ expression in vivo, we studied skeletal muscle from Goto Kakizaki (GK) rats, a nonobese normolipidemic animal model of type 2 diabetes developed by selective breeding of glucose-intolerant Wistar rats over several generations. This animal model is characterized by hyperglycemia and peripheral insulin resistance, primarily due to a beta cell defect (Portha, 2005). Rats were treated for 4 weeks with phlorizin (phloretin-2'- β -glucoside), an inhibitor of renal glucose reabsorption to normalize glycemia, glucose tolerance, and insulin sensitivity (Krook et al., 1997; Song et al., 1999). Skeletal muscle DGK δ protein expression was decreased by 35% ($p < 0.05$) in GK rats versus nondiabetic Wistar rats (Figure 1D). In contrast, DGK ζ and α protein expression in GK rats was unaltered (Figure S2), consistent with our human study, highlighting that the DGK δ isoform is specifically downregulated in diabetes. Phlorizin treatment in diabetic GK rats normalized glycemia and restored DGK δ protein expression. Total DGK activity (Figure 1E) and DAG mass (Figure 1F) were measured. Total DGK activity was decreased and intracellular DAG mass was increased in skeletal muscle from diabetic GK rats, indicating that the DGK δ isoform is a major contributor to total DGK activity in rodent skeletal muscle. Normalization of glycemia in phlorizin-treated GK rats restored skeletal muscle DGK activity and DAG mass. Conversely, liver expression of DGK δ and DAG mass was similar between Wistar and GK rats treated with either vehicle or phlorizin (Figure S3). In fat-fed Wistar rats, which display peripheral insulin resistance and glucose intolerance but not hyperglycemia, skeletal muscle DGK δ protein expression was unaltered (Figure 1H). Thus, hyperglycemia directly influences DGK δ protein expression, DGK activity, and DAG mass in skeletal muscle but not liver.

Inhibition of DGK Activity Decreases Glucose Transport through a DAG-cnPKC Pathway

Alterations in skeletal muscle DGK activity change the local concentration of DAG and PA, which may subsequently influence insulin signaling and metabolism. We determined whether inhibition of DGK alters insulin action in intact rat *epitrochlearis* muscle using two pan-DGK class I inhibitors, R59949 or R59022 (Jiang et al., 2000). These inhibitors bind to the catalytic domain of DGK isoforms and inhibit kinase activity. Incubation of rat *epitrochlearis* muscle with R59949 or R59022 decreased total DGK activity (Figure 2A) and increased DAG levels (Figure 2B), highlighting an inverse relationship between DGK activity and DAG accumulation in skeletal muscle. In parallel with the DAG accumulation, conventional/novel PKC (cnPKC) activity

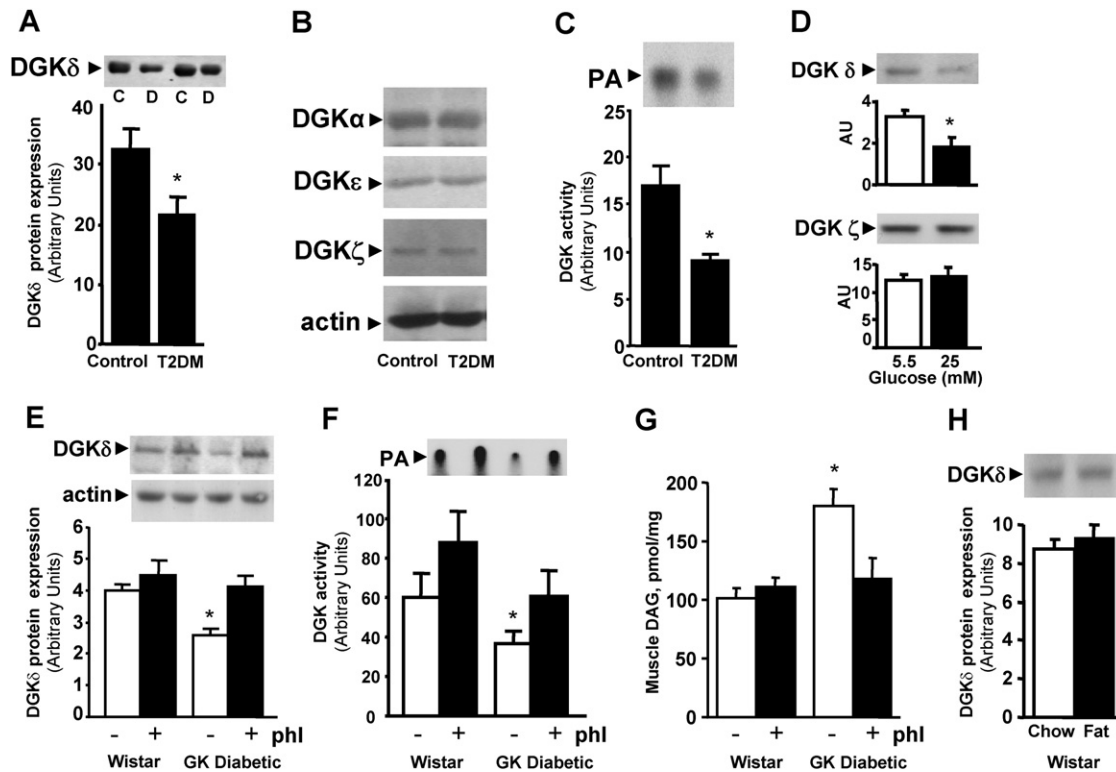


Figure 1. Skeletal Muscle DGKδ Protein Expression and DGK Activity Is Reduced in Type 2 Diabetic Patients and Hyperglycemic Diabetic GK Rats

(A and B) Skeletal muscle lysates from type 2 diabetic ($n = 11$) and nondiabetic ($n = 11$) participants were subjected to immunoblot analysis using an antibody against DGKδ (A). Upper panel is representative image and lower panel is mean \pm SEM, arbitrary units. * $p < 0.05$ versus control. Representative immunoblot analysis (B) for DGKα, DGKε, DGKζ, and actin (loading control) is shown.

(C) Total DGK activity in skeletal muscle homogenates from type 2 diabetic and controls. Upper panel is representative autoradiogram of phosphatidic acid (PA) production, lower panel mean \pm SEM, arbitrary units.

(D) DGKδ and DGKζ protein expression in primary human myotubes exposed to 5.5 or 25 mM glucose. * $p < 0.05$ versus 5.5 mM glucose.

(E–G) *Gastrocnemius* muscle from Wistar and GK rats after 4 week treatment with phlorizin (phl) or saline ($n = 5$ –6 rats). Shown are DGKδ and actin protein expression (E), Total DGK activity (F), DAG content (G). * $p < 0.05$ versus Wistar.

(H) DGKδ protein in *gastrocnemius* muscle from Wistar rats fed chow or high-fat diet for 4 weeks. * $p < 0.05$ versus chow-fed.

For (D)–(H), upper panel is representative image and lower panel mean \pm SEM, arbitrary units.

(Figure 2C) and isoform-specific phosphorylation of PKCδ (Figure 2D) were increased. DGK inhibition did not alter IR tyrosine phosphorylation (Figure 2E) but increased IRS-1 Ser307 and Ser302 phosphorylation (Figures 2F and 2G), implicating DAG-induced PKC activity in the negative regulation of insulin signaling. Indeed, insulin action on IRS-1 tyrosine phosphorylation, IRS-1-associated phosphatidylinositol (PI) 3-kinase activity, and Akt/PKB kinase phosphorylation was impaired in muscle exposed to the DGK inhibitors (Figure 2H). DGK inhibition also reduced insulin-stimulated glucose transport in isolated rat *epitrochlearis* muscle (Figures 2I and 2J). To directly explore a role of DAG-induced PKC-mediated insulin resistance, muscles were coincubated with R59949 and the broad spectrum PKC inhibitor GF109203X (Figures 2H and 2I). Under these conditions, insulin action on IRS-1, PI 3-kinase, Akt, and glucose transport was restored. These results implicate increased DAG levels and PKC activity in the regulation of the insulin response.

To address whether the effect of DGK inhibitors on glucose transport is due to reduced insulin signaling or downstream

effects on GLUT4 trafficking or activity, *epitrochlearis* muscle was exposed to hypoxia (Figure 2J), an insulin-independent mediator of GLUT4 translocation and glucose transport (Cartee et al., 1991). Hypoxia-mediated glucose transport was unaltered in muscle coincubated with R59949; thus DGK inhibition does not appear to directly alter GLUT4 traffic. Moreover, GLUT4 protein expression was unaltered in muscle exposed to DGK inhibitors (data not shown).

To exclude a possible direct inhibitory effect of R59949 on other kinases along the insulin signaling pathway, a specificity test of the R59949 was performed against a panel of 30 protein kinases in an in vitro assay (Bain et al., 2003; Davies et al., 2000). Among these kinases, only serum and glucocorticoid regulated kinase (SGK) was considerably inhibited by R59949 (Figure S4). The specificity assays were conducted at an ATP concentration far lower than that which is present in cells. Since R59949 is likely to be an ATP competitive inhibitor, this compound is expected to be a far weaker inhibitor of SGK1 in cells. Indeed, phosphorylation of NDRG1, a specific substrate of SGK expressed in skeletal

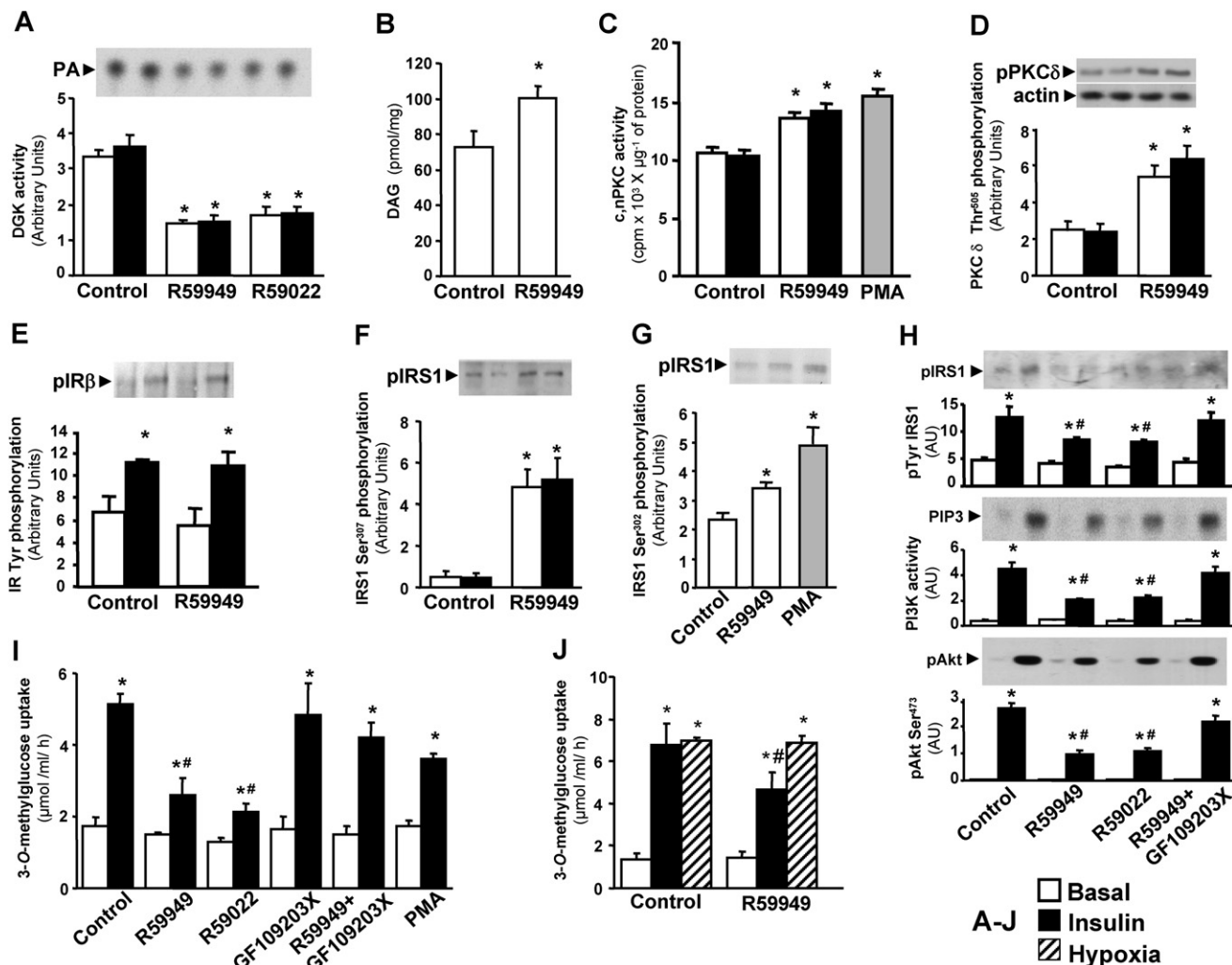


Figure 2. DGK Inhibition Attenuates Insulin Action in Rat Muscle

Isolated epitrochlearis muscle ($n = 6-11$) was incubated in the absence or in presence of 2.4 nM insulin and/or 25 μ M R59949, 50 μ M R59022, and/or 1 μ M of PMA. Muscle homogenates were prepared for (A) total DGK activity, (B) DAG content, and (C) combined conventional and novel PKC activity analysis. Lysates were subjected to immunoblot analysis for (D) PKC δ Thr505 phosphorylation, (E) IR tyrosine phosphorylation, (F) IRS-1 Ser307 phosphorylation, and (G) IRS-1 Ser302 phosphorylation. Skeletal muscle was also incubated (H-I) in the absence or presence of 1 μ M PMA, 1 μ M GF109203X, 25 μ M R59949, or 50 μ M R59022. (H) IRS-1 tyrosine phosphorylation, PI 3-kinase activity, Akt Ser473 phosphorylation, and (I-J) 3-O-methyl-glucose transport were assessed. Upper panel is representative image. Lower panel is mean \pm SEM, * $p < 0.05$ versus non-insulin-stimulated control. # $p < 0.05$ versus insulin-stimulated.

muscle (Murray et al., 2004), was unaffected by the concentrations of R59949 used in this study. Moreover, skeletal muscle SGK protein expression was unaltered between type 2 diabetic versus control subjects, or Wistar versus diabetic GK rats. Addition of R59949 directly to the assay buffer did not alter PI 3-kinase activity (Figure S4). Thus, the impairment in insulin signaling and glucose uptake is likely a direct consequence of DGK inhibition.

DGK Protein Expression and Activity in DGK δ Haploinsufficient Mice

As there are several DGK isoforms expressed in skeletal muscle (α , δ , ϵ , and ζ), we cannot completely resolve whether chemical inhibitors of DGK activity will confer the same phenotype as DGK δ isoform-specific inhibition. The DGK activity inhibited by

either R59949 or R59022 is primarily derived from class I DGK isoforms (Jiang et al., 2000). Although the DGK isoforms inhibited by either R59949 or R59022 appear to affect insulin signaling and glucose uptake, only DGK δ , a class II DGK isoform, was reduced in type 2 diabetic patients and diabetic GK rats. To determine whether the reduction in DGK δ protein expression in skeletal muscle from type 2 diabetic patients is of direct physiological relevance for altered glucose homeostasis, we metabolically characterized heterozygous DGK δ whole-body knockout mice. DGK $\delta^{+/-}$ mice were generated as described (Crotty et al., 2006). Inhibition of DGK activity by R59949 in skeletal muscle homogenates from either Wistar and GK rats or wild-type (WT) and DGK $\delta^{+/-}$ mice indicates that the DGK δ isoform accounts for approximately 60% of total DGK activity (Figure S5).

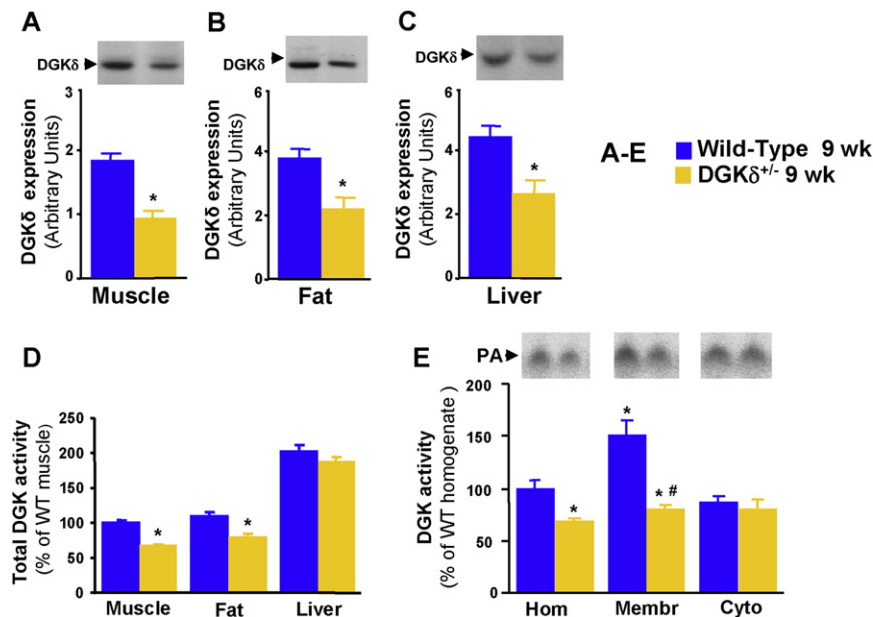


Figure 3. DGK Protein Expression and Activity in DGK δ Haploinsufficient Mice

(A–C) DGK δ protein expression in (A) *gastrocnemius* muscle, (B) epididymal fat, and (C) liver in DGK δ ^{+/-} mice versus WT mice at 9 weeks of age. (D) Total DGK activity, per gram of tissue protein, in homogenates prepared from *gastrocnemius* muscle, epididymal fat, and liver. (E) Total DGK activity per gram of tissue protein in homogenate (Hom), crude membrane fraction (Membr), and cytoplasmic (Cyto) fraction isolated from *gastrocnemius* muscle from 9-week-old DGK δ ^{+/-} mice. Results are mean ± SEM for n = 7–15 animals. *p < 0.05 versus 9-week-old WT mice.

DGK δ ^{+/-} mice have a 50% reduction in DGK δ protein in skeletal muscle (Figure 3A), which is similar to the 37% reduction in skeletal muscle DGK δ protein expression in type 2 diabetic patients (Figure 1A). DGK δ protein expression in fat and liver of DGK δ ^{+/-} mice was also reduced (Figures 3B and 3C). Total DGK activity was reduced in skeletal muscle and fat but not liver (Figure 3D). Total DGK activity in homogenates and a crude membrane fraction obtained from *gastrocnemius* muscle from DGK δ ^{+/-} mice was reduced, while DGK activity in the cytoplasmic fraction was unaltered (Figure 3E). The decrease in DGK activity in the membrane fraction in DGK δ ^{+/-} mice highlights the potential importance of the membrane localization of DGK δ signaling. DGK δ contains a PH domain and has been found in endoplasmic reticulum and Golgi apparatus (Nagaya et al., 2002). Subcellular fractionation of rat *gastrocnemius* muscle revealed that DGK δ protein is mainly associated with intracellular membranes (data not shown).

DGK δ Haploinsufficiency Leads to Development of Glucose Intolerance and Obesity

Age is an important risk factor for the development of skeletal muscle insulin resistance and type 2 diabetes (Nair, 2005). Thus, DGK δ ^{+/-} mice were studied at 9 or 36 weeks. Blood chemistry for DGK δ ^{+/-} and WT littermates is presented (Table S3). At 9 weeks, body weight and epididymal fat pad weight were unaltered in DGK δ ^{+/-} mice (Figure 4A). However, at 36 weeks, body weight and epididymal fat pad weight were increased in DGK δ ^{+/-} mice, concomitant with increase in plasma leptin concentration (Table S3). Plasma resistin concentration was unaltered in DGK δ ^{+/-} mice but significantly increased at 36 week regardless of genotype (Table S3). Plasma adiponectin levels were unaltered between DGK δ ^{+/-} and WT mice (Table S3).

An intraperitoneal glucose tolerance test (IPGTT) was performed at 9 and 36 weeks. Glucose tolerance, fasting blood glucose, and plasma insulin levels were normal at 9 weeks in DGK δ ^{+/-} mice (Figures 4B and 4C and Table S3). In contrast, glucose tolerance

was significantly impaired at 36 weeks in DGK δ ^{+/-} mice (Figures 4B and 4C), compared with age-matched littermates. In older WT mice, insulin levels in the fasting state, as well as during the IPGTT, were also elevated compared to younger WT mice, indicative of development of insulin resistance. Glucose tolerance was impaired and insulin levels were elevated in DGK δ ^{+/-} mice compared to age-matched littermates. These age-associated changes in glucose homeostasis were independent of any further reduction in DGK δ expression or total DGK activity (Figures 4D and 4E). Skeletal muscle DAG levels were increased by 40% in DGK δ ^{+/-} mice (Figure 4F), underscoring the contribution of DGK δ to skeletal muscle DAG homeostasis. In contrast, DAG levels were unaltered in liver of DGK δ ^{+/-} mice (Figure S6), indicating that this isoform plays a minor role in total DGK activity in liver.

Insulin-stimulated glucose transport was assessed in isolated *soleus* muscle at 9 and 36 weeks in WT and DGK δ ^{+/-} mice (Figure 4G). Despite normal fasting insulin, glucose tolerance, and body weight in DGK δ ^{+/-} mice at 9 weeks, insulin-stimulated glucose transport in response to 0.36 nM, but not 120 nM, was decreased in isolated *soleus* muscle. Moreover, the impairment in skeletal muscle insulin-stimulated glucose transport was observed prior to the development of obesity, hyperinsulinemia, or glucose intolerance. An impairment in submaximal (0.36 nM), but not maximal (120 nM), insulin-stimulated glucose transport was noted in WT mice at 36 weeks. In DGK δ ^{+/-} mice, basal glucose transport was unaltered at 36 weeks, but insulin-stimulated glucose transport was impaired, concomitant with obesity, hyperinsulinemia, and glucose intolerance, compared to age-matched WT littermates. Skeletal muscle GLUT4 protein expression was unaltered between WT and DGK δ ^{+/-} mice.

Consequence of DGK δ Haploinsufficiency on Peripheral Insulin Sensitivity and Signaling

Since glucose uptake in skeletal muscle and adipose tissue accounts for the majority of peripheral glucose disposal, we examined tissue-specific responses for glucose uptake in vivo during a euglycemic-hyperinsulinemic clamp in conscious mice. We studied 9-week-old mice to avoid the potential deleterious effect of hyperinsulinemia and/or obesity on whole-body insulin-mediated glucose homeostasis, as these factors

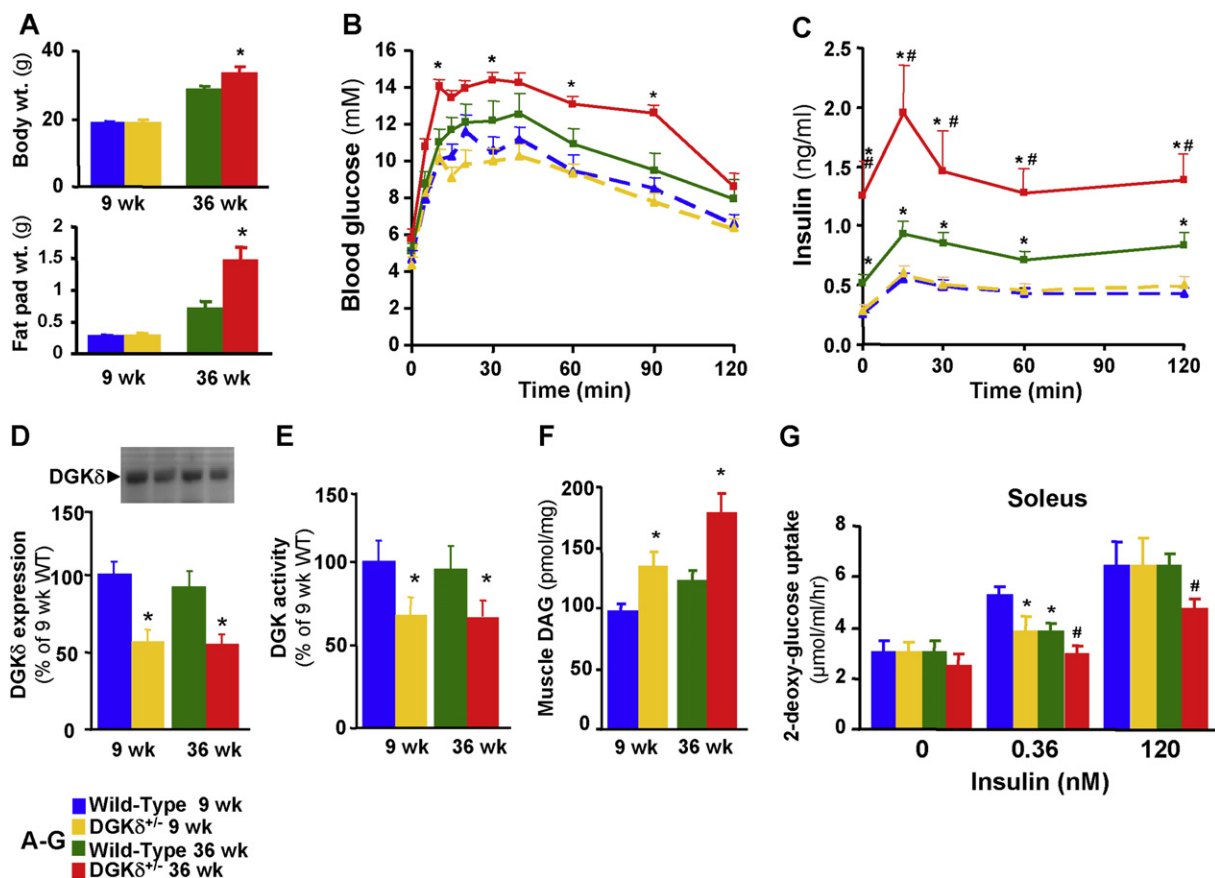


Figure 4. DGK δ Haploinsufficiency Leads to Glucose Intolerance and Obesity

(A) Body weight and epididymal fat pad weight of DGK $\delta^{+/-}$ and WT mice at 9 and 36 weeks.

(B and C) Glucose (B) and insulin (C) during glucose tolerance in overnight fasted mice.

(D) Skeletal muscle lysates were subjected to immunoblot analysis using an antibody against DGK δ . Upper panel is representative image and lower panel is mean \pm SEM, arbitrary units. * $p < 0.05$ versus WT.

(E and F) Total DGK activity (E) and DAG content (F) in skeletal muscle homogenate. Results are mean \pm SEM.

(G) Basal and insulin-stimulated in vitro 2-deoxyglucose uptake in isolated soleus muscle from WT and DGK $\delta^{+/-}$ mice. Results are mean \pm SEM for 8–12 mice.

* $p < 0.05$ versus WT mice at 9 weeks, # $p < 0.05$ versus WT mice at 36 weeks.

independently contribute to insulin resistance. Euglycemic-hyperinsulinemic clamps were performed in separate experiments using either a low (1.25 mU/min/kg; Figure 5A) or high (10 mU/min/kg; Figure 5B) dose insulin infusion. Under the low-dose insulin infusion (Figure 5A), plasma insulin levels were increased 2-fold (0.2 ± 0.1 versus 0.4 ± 0.06 ng/ml). Insulin-mediated peripheral glucose utilization was increased 66% versus 18% over basal in WT versus DGK $\delta^{+/-}$ mice, respectively, whereas the hepatic insulin response was similar between genotypes. Under the high dose insulin infusion (Figure 5B), plasma insulin levels were markedly increased (0.4 ± 0.1 versus 5.2 ± 0.4 ng/ml), and hepatic glucose output was completely inhibited in both genotypes. Similar to results for the low-dose insulin infusion, peripheral glucose utilization was impaired in DGK $\delta^{+/-}$ mice.

In vivo tissue-specific glucose uptake was also determined in separate experiments under the high-dose insulin infusion (Figures 5C and 5D). In DGK $\delta^{+/-}$ mice, insulin-stimulated glucose uptake (Figure 5C) was significantly decreased in EDL (34%)

and soleus (21%) and tended to be decreased in gastrocnemius (36%, $p = 0.09$), diaphragm (36%, $p = 0.12$), and heart (29%, $p = 0.07$) muscle. Insulin-mediated glucose uptake was also significantly decreased in white adipose tissue (50%) but not brown adipose tissue (BAT) (Figure 5D). The impaired skeletal muscle glucose uptake was associated with reduced insulin-stimulated tyrosine phosphorylation of IR and IRS-1 and serine/threonine phosphorylation of Akt and AS160, components of the canonical insulin signaling cascade important for glucose uptake and metabolism (Figure 5E). In contrast, liver insulin-stimulated phosphorylation of IR, IRS-1, IRS-2, and Akt was similar between DGK $\delta^{+/-}$ and WT mice (Figure 5F). Thus, DGK $\delta^{+/-}$ mice develop insulin resistance in skeletal muscle and adipose tissue, but not liver, prior to development of obesity, glucose intolerance, and hyperinsulinemia. Since defects in glucose uptake were observed in isolated skeletal muscle, as well as at the whole-body level, DGK δ expression directly influences skeletal muscle insulin sensitivity independent of alterations in the metabolic milieu.

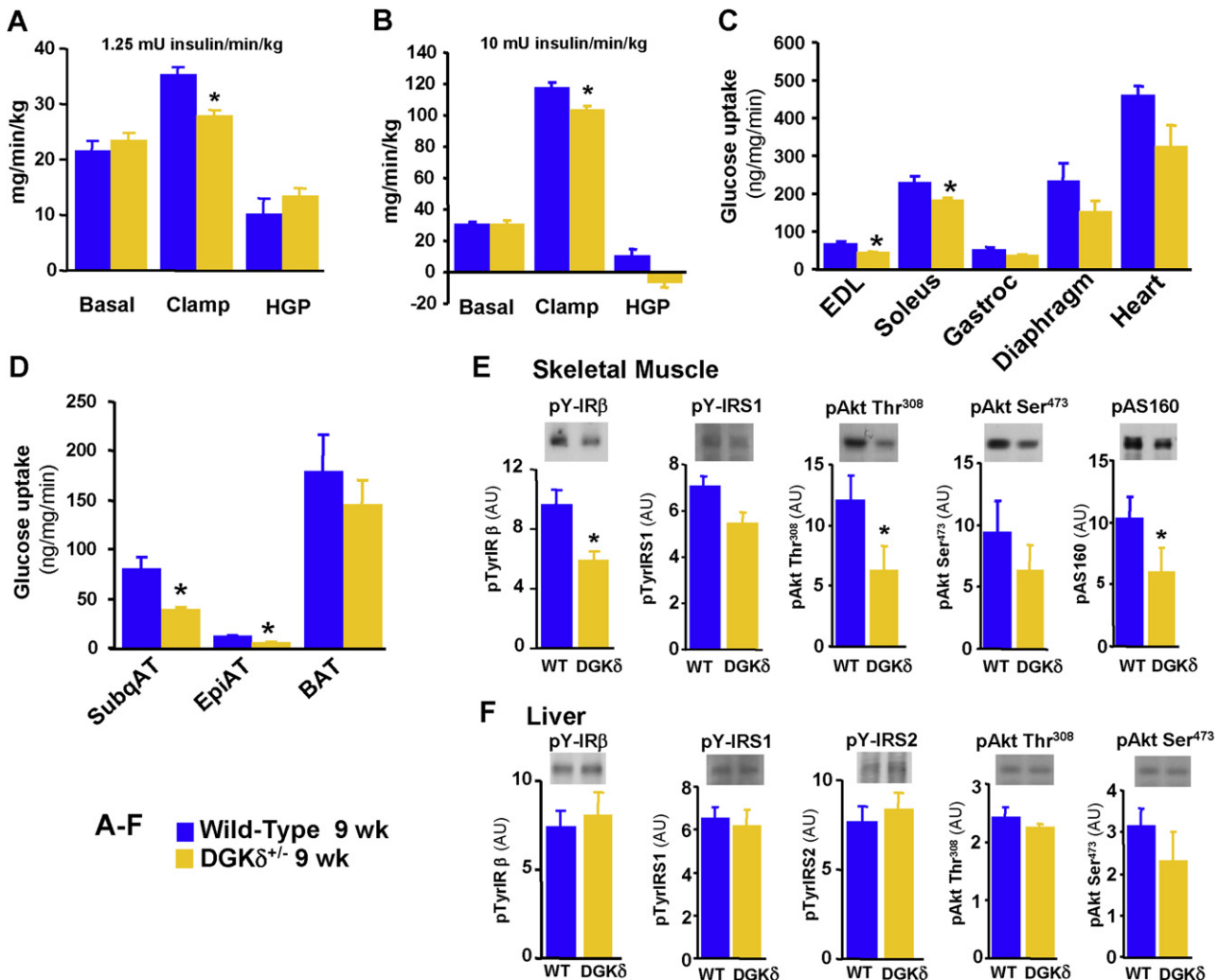


Figure 5. DGKδ Haploinsufficiency Impairs Peripheral Insulin Sensitivity and Signaling

(A and B) In vivo glucose utilization during euglycemic-hyperinsulinemic clamp under low (A; 1.25 mU/min/kg) or high (B; 10 mU/min/kg) dose insulin infusion in 9-week-old DGKδ^{+/-} and WT mice.

(C and D) In vivo insulin-stimulated glucose uptake in (C) skeletal and cardiac muscle (D) and subcutaneous (SubqAT), epididymal (EpiAT), and brown adipose tissues (BAT) after high-dose insulin infusion.

(E and F) Insulin signal transduction in skeletal muscle (E) or liver (F) measured after high-dose insulin infusion. Upper panel is representative image and lower panel is mean ± SEM, arbitrary units. Results are mean ± SEM for n = 7–9 mice. *p < 0.05 versus WT mice.

Skeletal Muscle Lipid Oxidation and Whole-Body Energy Homeostasis Are Altered in DGKδ^{+/-} Mice

We next determined whether DGKδ expression influences skeletal muscle lipid metabolism or whole-body energy homeostasis. Skeletal muscle triglyceride content and plasma nonesterified fatty acid were unaltered between genotypes (Table S3); however, oleate oxidation was reduced 30% in isolated soleus muscle from 9-week-old DGKδ^{+/-} mice (Figure 6A). Whole-body energy homeostasis was also determined through analysis of food intake, locomotor activity, oxygen consumption, and respiratory exchange ratio (RER). Although food intake and locomotor activity did not differ between genotypes over a 24 hr period (Figures 6B and 6C), oxygen consumption (VO₂; ml/kg/hr) was reduced in DGKδ^{+/-} mice (Figure 6D). RER was determined

in the light and dark cycles, when animals were in a rested and active state, respectively. DGKδ^{+/-} mice failed to appropriately shift between lipid and glucose oxidation in the transition between the light (rested) and dark (active) states (Figure 6E), thereby demonstrating “metabolic inflexibility.” Changes in skeletal muscle oleate oxidation and whole-body energy homeostasis were observed prior to the development of obesity.

Signaling Impairments in Skeletal Muscle from DGKδ^{+/-} Mice

Elevations in DAG have been linked to increased PKC activity and serine phosphorylation of IRS-1, constituting a negative feedback regulation along insulin signaling cascades governing glucose and lipid metabolism (Itani et al., 2002; Yu et al.,

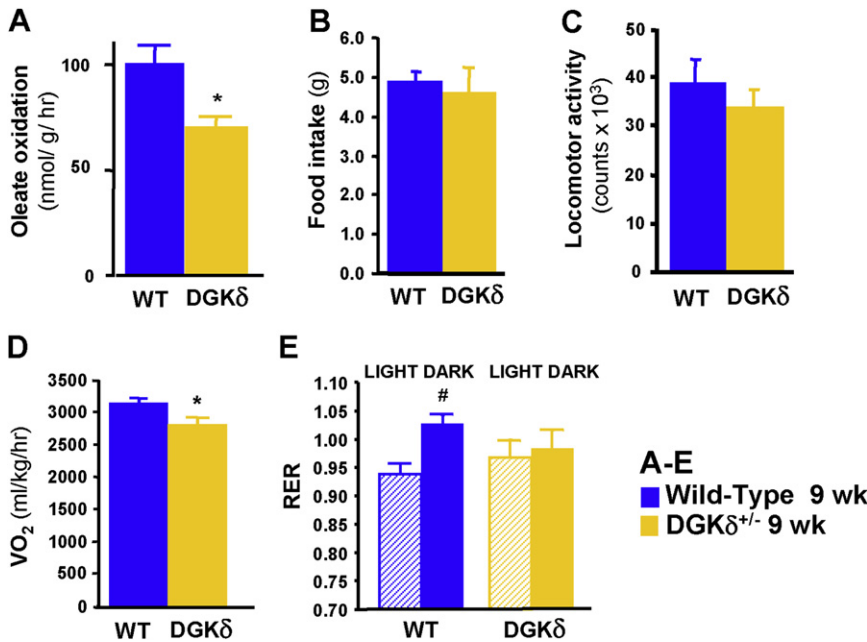


Figure 6. Skeletal Muscle Lipid Oxidation and Whole-Body Energy Homeostasis Impairments in DGK $\delta^{+/-}$ Mice

(A) Oleate oxidation in isolated *soleus* muscle from fed DGK $\delta^{+/-}$ and WT mice.
 (B) Food intake assessed over 24 hr.
 (C) Locomotor activity assessed over 24 hr.
 (D) Whole-body oxygen consumption (VO₂) assessed over 24 hr.
 (E) Respiratory exchange ratio assessed over 24 hr, during the light (sedentary) and dark (active) cycles.
 Results are mean \pm SEM, *p < 0.05 versus WT mice.

2002). Expression and phosphorylation of DAG-dependent PKCs and IRS-1 were determined in skeletal muscle (Figure 7) and liver (Figure S6) from DGK $\delta^{+/-}$ mice and WT littermates. Phosphorylation and expression of PKC θ and η were unaltered in skeletal muscle from DGK $\delta^{+/-}$ mice. Phosphorylation of PKC δ was increased in skeletal muscle from DGK $\delta^{+/-}$ mice (Figure 7A), similar to the effect of DGK inhibition by R59949. Protein expression of IR and IRS-1 (Figures 7B and 7C) was reduced in DGK $\delta^{+/-}$ mice at 9 weeks but not 36 weeks. IR and IRS-1 expression in both genotypes at 36 weeks was lower compared to 9 weeks. IRS-1 Ser307 phosphorylation normalized to the level of IRS-1 protein expression was increased in DGK $\delta^{+/-}$ mice (Figure 7D).

the observation that peripheral, but not hepatic, insulin sensitivity was impaired. Collectively these data indicate that a reduction in DGK δ impairs insulin signaling in skeletal muscle.

Genetic Analysis of DGKD

We analyzed quantitative traits for the *DGKD* gene in 234 offspring of type 2 diabetic patients who had a nondiabetic oral glucose tolerance test. One of the parents had to have type 2 diabetes and the other had to exhibit normal glucose tolerance. Nine single-nucleotide polymorphisms (SNPs) properly covering the promoter and coding regions of *DGKD* were screened. Different SNPs had a nominal association (p < 0.05) with insulin

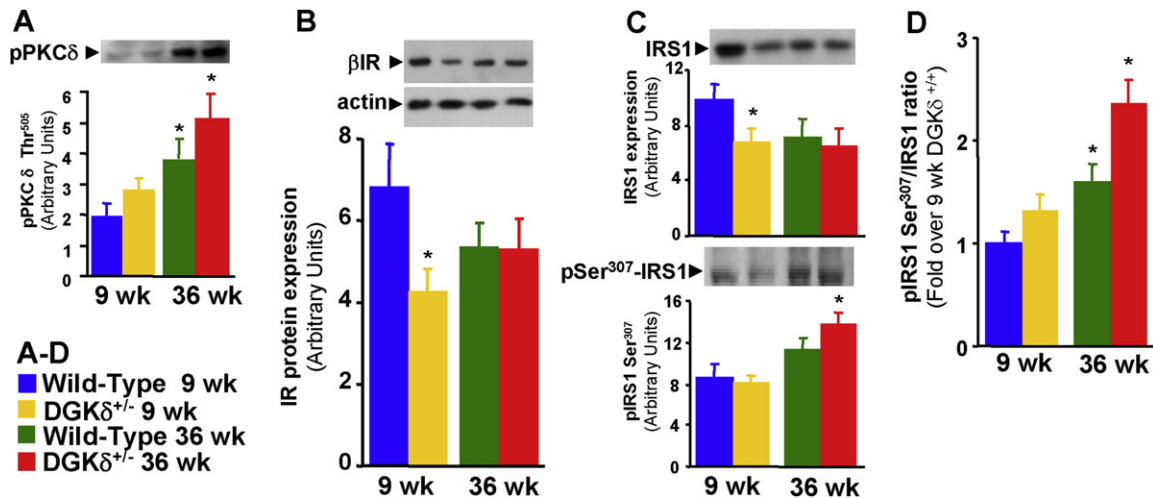


Figure 7. Signaling Impairments in Gastrocnemius Muscle from DGK $\delta^{+/-}$ Mice

(A–D) Muscle lysates were subjected to immunoblot analysis for (A) PKC δ Thr505 phosphorylation, (B) IR protein and actin (loading control), (C and D) IRS-1 protein/IRS-1 Ser307 phosphorylation in WT mice and DGK $\delta^{+/-}$ mice at 9 and 36 weeks. Upper panel shows representative image. Graphs are mean \pm SEM, *p < 0.05 versus 9-week-old WT mice.

area under the curve (AUC) in a 2 hr oral glucose tolerance test, glucose AUC during the intravenous glucose tolerance test (IVGTT), and insulin AUC from 10 to 60 min in an IVGTT (Table S4). No association was observed between *DGKD* SNPs and insulin sensitivity, as measured by the euglycemic clamp.

DISCUSSION

Intracellular DAG levels are elevated in skeletal muscle from insulin-resistant rodents and humans (Itani et al., 2002; Kraegen et al., 2006), suggesting a role for lipid intermediates in the development of insulin resistance. DGK isoforms modulate the balance between DAG and PA levels and terminate DAG signals through the conversion of DAG to PA (Sakane et al., 2007; Topham, 2006). DGK isoforms interact with specific isoforms for PKC, RasGRP, and PI kinases to regulate numerous biological events including cell growth, neuronal transmission, and cytoskeleton remodeling (Sakane et al., 2007; Topham, 2006). We reveal a previously unrecognized role for DGK δ in contributing to hyperglycemia-induced insulin resistance and impairments in energy homeostasis, thereby exacerbating the severity of type 2 diabetes. Reduced DGK δ activity, either by chemical inhibition or by genetic ablation of the enzyme using DGK $\delta^{+/-}$ mice, caused aberrant insulin signaling, glucose uptake defects in skeletal muscle and adipose tissue, impaired whole-body insulin resistance, and obesity. Our findings in DGK $\delta^{+/-}$ mice demonstrate that reduced DGK δ expression directly causes peripheral insulin resistance, metabolic inflexibility, mild obesity, and further metabolic derangements.

Skeletal muscle protein expression of DGK δ and total DGK activity was reduced in type 2 diabetic patients and diabetic rodents and normalized upon correction of hyperglycemia. This finding strongly suggests that DGK δ undergoes downregulation as a consequence of the altered metabolic milieu. Indeed DGK δ , but not α , ϵ , or ζ , protein expression was negatively correlated with fasting glucose and HbA_{1c}. These defects were localized to peripheral, rather than hepatic, tissues. Although variations at the *DGKD* locus are unlikely to constitute a major genetic cause of type 2 diabetes (Saxena et al., 2007; Sladek et al., 2007), we found suggestive evidence that SNPs of *DGKD* are associated with altered glucose metabolism in offspring of type 2 diabetic subjects. DGK δ appears to play an important role in metabolic regulation since environmental factors can also contribute to regulation of insulin sensitivity in type 2 diabetes. Exercise training-induced improvements in insulin sensitivity have been positively correlated with an increase in skeletal muscle DGK δ gene expression in nondiabetic (Teran-Garcia et al., 2005) and type 2 diabetic (Fritz et al., 2006) people, highlighting a potential role of this enzyme in the regulation of metabolism in response to regular exercise. Rare genetic defects in *DGKD* have been observed; a recent case study of a 12-year-old-female with epilepsy provides genetic support that disruption on *DGKD* is associated with seizures, capillary abnormality, developmental delay, infantile hypotonia, and obesity (Leach et al., 2007). While the DGK $\delta^{+/-}$ mice studied here also displayed an obesity phenotype with aging, they did not display any obvious growth defects or sign of seizures.

Insulin sensitivity is impaired in younger DGK $\delta^{+/-}$ mice that are not obese and have yet to show any signs of hyperglycemia,

glucose intolerance, systemic hyperinsulinemia, or alterations in food intake and locomotor activity. DGK δ haploinsufficiency can cause “primary” peripheral insulin resistance, impaired lipid oxidation, and impaired energy homeostasis, which leads to obesity in later life. Insulin-mediated glucose uptake and uncoupling protein 1 protein expression in BAT were unaltered between genotypes, providing evidence against metabolic alterations in BAT as a cause of the obesity phenotype in DGK $\delta^{+/-}$ mice. Furthermore, hypothalamic mRNA expression of neuropeptides including neuropeptide Y, proopiomelanocortin, and agouti-related protein is unaltered in DGK $\delta^{+/-}$ mice, despite mild obesity and insulin resistance with aging. These results do not preclude an effect of DGK δ in energy homeostasis via actions in other tissues.

The reduction in DGK δ expression and activity appears to promote peripheral insulin resistance and metabolic inflexibility, which may contribute to the obesity phenotype. Metabolic inflexibility, as defined by an inability to appropriately transition between lipid and carbohydrate oxidation, has been linked to impaired oxidative capacity in insulin resistance and type 2 diabetes (Storlein et al., 2004). Impaired oleate oxidation and peripheral insulin-mediated glucose uptake in DGK $\delta^{+/-}$ mice were associated with increased adipose mass and increased body weight, which further contributed to the development of glucose intolerance. These defects may arise from alterations in mitochondrial size and density, expression of oxidative enzymes, or changes in skeletal muscle fiber type. Therefore, DGK $\delta^{+/-}$ mice resemble many of the features associated with the clinical manifestation of peripheral insulin resistance in type 2 diabetes in humans.

Disruption of insulin signaling events associated with GLUT4 translocation impairs glucose transport (Ishiki and Klip, 2005). Based on our clinical and experimental studies, we describe a signal transduction pathway involving DAG-induced insulin resistance as a result of impaired DGK δ expression and activity. In addition to a local increase in DAG, a decrease in DGK δ expression and DGK activity leads to a concomitant decrease in PA levels. DGK enzymes act as both terminators of DAG-mediated signals and activators of PA-mediated signals. Protein targets of PA include positive regulators of glucose transport, such as PKC ζ , and membrane vesicle traffic, such as phosphatidylinositol 4-phosphate 5-kinase (Sakane et al., 2007; Topham and Prescott, 1999). Interestingly, insulin-independent glucose transport was unaltered by DGK inhibition. Nevertheless, we cannot exclude the possibility that the reduction of DGK δ contributes to the regulation of glucose transport through regulating the production of PA.

The increase in DAG mass and PKC activity offers a possible mechanism for the decreased insulin signaling in DGK $\delta^{+/-}$ mice. The impairment in whole-body insulin-mediated glucose uptake may be attributed to DAG accumulation, elevated PKC activity, and serine phosphorylation of IRS-1, leading to peripheral insulin resistance and impaired energy homeostasis. Reduced membrane-associated DGK activity may change subcellular localization and compartmentalization of important signal transducers and contribute to an altered metabolic response. Confirming an effect of DGK δ deficiency on tyrosine receptor-mediated signaling, DAG-induced PKC activation increases

epidermal growth factor receptor threonine phosphorylation and reduces receptor expression and activity in keratinocytes from DGK δ null mice (Crotty et al., 2006). This provides a link between DGK δ , PKCs, and consequently EGFR and insulin signaling pathways. Interestingly, differences between chemical inhibition of DGK and heterozygosity for DGK δ with respect to IR expression and phosphorylation were noted, suggesting that reduced IR protein expression in DGK $\delta^{+/-}$ mice may arise from chronic transcriptional effects as observed for EGFR in DGK δ null mice. Our results cannot exclude the possibility that reduced IR expression contributes to the observed impairments in insulin sensitivity and glucose homeostasis in DGK $\delta^{+/-}$ mice. Muscle-specific IR dominant-negative transgenic mice (Chang et al., 1994; Moller et al., 1996) or IR null mice (Bruning et al., 1998) develop mild obesity secondary to defects in skeletal muscle insulin action.

Reduced DGK δ protein expression directly contributes to the development of peripheral insulin resistance and mild obesity. Data from DGK $\delta^{+/-}$ mice support this concept, as a 50% reduction in DGK δ protein expression induces peripheral insulin resistance and mild obesity in the absence of hyperglycemia. Reduced DGK δ protein expression can act as a primary mediator of insulin resistance in type 2 diabetes and promote mild obesity with aging. Data from diabetic GK rats also provide evidence that insulin resistance occurs in concert with hyperglycemia-induced downregulation of DGK δ . In this case, the reduction in DGK δ protein occurs in response to hyperglycemia or other environmental factors, which secondarily impact DGK δ expression and DAG metabolism. Collectively, our findings highlight a role for DGK δ in the regulation of hyperglycemia-induced impairments in peripheral insulin sensitivity and reveal new insight into how to resolve the metabolic complexities associated with type 2 diabetes mellitus. Therapeutic approaches to target DAG and PA metabolism via regulation of DGK δ may control and prevent insulin resistance in metabolic disease.

EXPERIMENTAL PROCEDURES

Human Studies for Muscle Analysis

Subjects were matched for age, BMI, and physical fitness. Skeletal muscle biopsies were obtained from the *vastus lateralis* portion of the *quadriceps* muscle from 11 type 2 diabetic and 11 healthy control subjects (Table S2) with approval from the institutional ethical committee of Karolinska Institutet. Studies were performed according to the declaration of Helsinki and informed written consent was obtained. Type 2 diabetic subjects were treated with diet, sulfonylureas, or metformin. Individuals taking beta-adrenergic receptor blockers, ACE inhibitors, or hormonal therapy were excluded from the study. Skeletal muscle lysates were prepared and subjected to immunoblot analysis as described (Krook et al., 2000).

Subjects and Phenotype Analysis for Genetic Studies

The aim of this study was to investigate the association of SNPs of *DGKD* with parameters of glucose metabolism. The collection of subjects and the study protocol for our offspring study have been previously published (Salmenniemi et al., 2004), and a summary is available (Supplemental Experimental Procedures). The study protocol was approved by the Ethics Committee of the University of Kuopio. All subjects gave an informed consent. The mean age and body mass index of the subjects was 35 years and 26 kg/m², respectively, and all had nondiabetic glucose tolerance. Subjects underwent the following studies: an OGTT (n = 234), a euglycemic-hyperinsulinemic clamp (n = 217) to evaluate insulin sensitivity, and an intravenous glucose tolerance test

(IVGTT) (n = 217) to evaluate the first phase insulin secretion. Selection of SNPs of *DGKD* was based on the genotype data from Utah residents with ancestry from Northern and Western Europe available from HapMap project's website (Public Release #20, January 24, 2006) (Altshuler et al., 2005). Genotyping and statistical analysis are described (Supplemental Experimental Procedures).

Animal Studies

Animals were maintained in a temperature- and light-controlled environment and were cared for in accordance with regulations for the protection of laboratory animals. The regional animal ethical committee approved all experimental procedures. Animals were maintained under a 12 hr light:dark cycle and had free access to water and standard rodent chow.

Goto Kakizaki Rats and Treatment

Male GK and weight-matched Wistar rats (200–250 g) from Karolinska Institutet were treated with phlorizin (0.8 g/kg body weight per day; as 40% solution in propylene glycol) or vehicle (equal amounts of propylene glycol per kilogram) for 4 weeks (Krook et al., 1997). *Gastrocnemius* muscle and liver were dissected and frozen in liquid nitrogen. Plasma insulin and glucose tolerance was previously determined (Krook et al., 1997).

Epitrochlearis Muscle Incubation and Glucose Transport

Male Wistar rats (120–140 g) were obtained from B&K Universal, Sollentuna, Sweden. Rats were anesthetized with pentobarbital and *epitrochlearis* muscles were dissected free and incubated for insulin signaling and glucose transport. Muscles were preincubated for 30 min either with 50 μ M DGK inhibitor I R59022, 25 μ M DGK inhibitor II R59949, 1 μ M Phorbol-12-Myristate-13-Acetate (PMA), or broad spectrum PKC inhibitor GF109203X (1 μ M) (Calbiochem). Muscle was incubated for 30 min (Wallberg-Henriksson, 1987) in the absence or presence of 2.4 nmol/l insulin (Actrapid, Novo Nordisk) or incubated under a nitrogen gas phase (hypoxia) in the absence or presence of inhibitors. After incubation, muscles were frozen in liquid nitrogen for subsequent glucose transport and insulin signaling analysis. Glucose transport activity was assessed (Wallberg-Henriksson, 1987).

Generation of DGK δ Haploinsufficient Mice

The description of the methods used to generate haploinsufficient DGK δ (DGK $\delta^{+/-}$) mice is presented in Crotty et al. (2006).

Glucose Tolerance Test and Metabolic Analysis in DGK $\delta^{+/-}$ Mice

Glucose (1 g/kg body weight) was administered to fasted DGK $\delta^{+/-}$ mice and WT littermates by intraperitoneal injection (Supplemental Experimental Procedures). Plasma insulin, NEFA, adiponectin, leptin, and resistin were determined (Supplemental Experimental Procedures). 2-deoxy-glucose uptake and oleate oxidation were assessed in isolated mouse *soleus* muscle (Barnes et al., 2004). Triglyceride content was determined in mouse *gastrocnemius* muscle using a triglycerides/glycerol blanked kit (Roche, Stockholm, Sweden) and a Seronorm lipid (SERO A/S, Oslo, Norway) standard.

Whole-Body Energy Homeostasis in DGK $\delta^{+/-}$ Mice

Food intake, oxygen consumption, RER, and locomotor activity were measured using a Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH, USA), (Supplemental Experimental Procedures).

Euglycemic-Hyperinsulinemic Clamp in Conscious in DGK $\delta^{+/-}$ Mice

Glucose turnover rate (GTR) was measured in the basal state and during euglycemic and hyperinsulinemic conditions, using a constant infusion of [3 - 3 H]glucose (Supplemental Experimental Procedures). Basal glucose production and utilization were assessed. *Gastrocnemius* muscle and liver were immediately frozen in liquid nitrogen for insulin signaling analysis.

Insulin-Stimulated Tissue-Specific Glucose Uptake in DGK $\delta^{+/-}$ Mice

Animals were studied as described above for the Euglycemic-Hyperinsulinemic Clamp procedure. 2-deoxy-D-[1- 14 C]glucose was administered to estimate insulin-stimulated glucose uptake and metabolism in different tissues (Supplemental Experimental Procedures).

Analytical Procedures

Total DGK activity was determined (Lee et al., 1999) (Supplemental Experimental Procedures). Phosphorylation and expression of protein signaling intermediates and PI 3-kinase activity was assayed (Krook et al., 2000). Skeletal muscle and liver DAG content was determined by conversion to phosphorylation products by externally added DAG-kinase from *E. coli* in the presence of [γ - 32 P] ATP (Preiss et al., 1986). Total conventional and novel PKC activity was measured in muscle homogenates without addition of exogenous DAG (Kikkawa et al., 1983). The specificity of the DGK inhibitor was tested against a panel of 30 different protein kinases (Figure S4) (Davies et al., 2000).

Statistics

Differences between groups were determined by ANOVA with multiple comparisons with Fischer's post hoc analysis. Student's *t* test was used for comparisons when only two parameters were evaluated. Correlations were determined using Pearson's Correlation Analysis. Significance was accepted at $p < 0.05$.

Supplemental Data

Supplemental Data include Experimental Procedures, four tables, and six figures and can be found with this article online at <http://www.cell.com/cgi/content/full/132/3/375/DC1/>.

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